PATENT
Docket No.: **176/61411 (2-11141-03010)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) :	Wu et al.	)	Examiner:
		)	Michail A. Belyavskyi
Serial No. :	10/679,184	)	
		)	Art Unit:
Cnfrm. No. :	2775	)	1644
		)	
Filed :	October 3, 2003	)	
		)	
For :	THREE-DIMENSIONAL PERIPHERAL LYMPHOID ORGAN CELL CULTURES	)	

SECOND DECLARATION OF ANDREA BOTTARO, Ph.D., UNDER 37 C.F.R. § 1.132

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, ANDREA BOTTARO, Ph.D., pursuant to 37 C.F.R. § 1.132 declare:

1. I received a first-level doctorate ("Laurea") in Biology from the University of Torino, Italy, in 1987, and a Research Doctorate (Ph.D. equivalent) in Human Genetics from the University of Torino, Italy, in 1993.

2. I am currently an Associate Professor of Medicine, Microbiology and Immunology, and Oncology, at the School of Medicine and Dentistry, University of Rochester, Rochester, New York.

3. I am a co-inventor of the above-identified patent application.

4. I understand that the U.S. Patent and Trademark Office considers that WO 01/036589 to Wu et al. ("Wu"), WO 99/15629 to Pykett et al. ("Pykett"), and U.S. Patent No. 5,160,490 to Naughton et al. ("Naughton") each teach a method for culturing peripheral lymphoid organ cells by culturing the cells on a three-dimensional scaffolding, which is covered

or surrounded with culture medium, under conditions effective to generate and maintain mature and functional peripheral lymphoid organ cells, where the three-dimensional scaffolding allows cells in the culture medium to have cell to cell contact in three dimensions. I have reviewed Wu, Pykett, and Naughton and am presenting this declaration to demonstrate that these references do not teach a method for culturing peripheral lymphoid organ cells under conditions effective to generate and maintain mature and functional peripheral lymphoid organ cells.

5. The intrinsic differences between the lymphoid and accessory cell populations in bone marrow and lymph nodes are crucial to their respective functions, namely lymphopoiesis and the generation of antigen-specific adaptive immune responses. To highlight these differences, and test their effects on the outcome of immunization experiments in bioreactor culture systems, we performed flow cytometry analysis on fresh and cultured tonsil and bone marrow samples, and tested their responses to immunization to a common vaccine antigen, tetanus toxoid. The bone marrow samples were cultured in a bioreactor as described in Wu, while the tonsil samples were cultured in a bioreactor as described in our application. The results are attached hereto as Exhibit 1.

6. We performed flow cytometry on fresh (*i.e.*, uncultured) bone marrow and tonsil samples to evaluate their cell population profiles. Figure 1 of Exhibit 1 (enclosed) shows a comparison of these flow cytometry profiles. Both tissues display substantial proportions of B ($CD19^+$ (region 1)) and T ($CD3^+$ (region 2)) lymphoid populations (top panels); although the abundance of B lymphocytes is lower in the bone marrow samples, to the expense of both T cells and, more notably, non-lymphoid populations (myeloid cells as well as erythroid precursors). When analyzed for expression of IgD, a marker of naive, mature B cells, these constitute the largest population in the tonsil, but only a small fraction in the bone marrow (Figure 1, center panels). (It is noted that in the bone marrow $CD19^+$, IgD^+ mature B cells represent a rapidly transient population of recirculating peripheral cells that are introduced via the blood circulation, but do not home to the bone marrow or reside there *in situ*.)

7. To better characterize the B cell subsets in each organ, we stained the samples for the markers CD27 and CD38, which we analyzed after gating selectively on $CD19^+$ (B lymphoid) cells (Figure 1, bottom panels). This analysis revealed four cell populations: mature B cells ($CD38^-$, $CD27^-$ (region 1)), memory B cells ($CD38^-$, $CD27^+$ (region 3)), activated B cells in the tonsil ($CD38^+$, $CD27^+$ (region 2)), and, in the bone marrow, B cell precursors

($CD38^{+}$, $CD27^{-}$ (region 2)) and plasma cells ($CD38^{++}$, $CD27^{++}$ (region 4)). The latter population is predominantly detected in the bone marrow, the homing site for long-lived plasma cells generated in the periphery.

8. Figure 2 of Exhibit 1 shows the same type of analysis performed on tonsil and bone marrow samples cultured for two weeks in the bioreactor system. The most notable change compared to the fresh samples is the almost complete loss of the mature, naive B cell population in the bone marrow culture (center panels (region 1)). We believe that this is because the bone marrow does not provide a suitable microenvironment for the recirculating mature B cells to survive in culture. In contrast, IgD^{+} cells are readily maintained in the tonsil cultures (our experiments show that these cells can survive for 5–7 weeks in the system). The residual B lymphoid population in the bone marrow bioreactors is comprised predominantly of the long-lived plasma cells ($CD38^{++}$, $CD27^{++}$ (region 4)) highlighted in the fresh sample, while the other subsets (mostly precursor B cells) are comparatively under-represented.

9. The loss of the mature naive population, the cell pool from which the humoral immune response precursors are selected after antigen stimulation, has significant implications for the functional potential of bone marrow cultures. To test this, we immunized 2-week-old bone marrow and tonsil cultures with tetanus toxoid antigen ("TTC") and lipopolysaccharide as a broad-spectrum adjuvant. Two weeks after immunization, following an antigen boost, we measured the number of cells secreting IgM and IgG antibodies specific to TTC using the Elispot technique. As shown in Figure 3 of Exhibit 1, in tonsil samples, a 2- to >5-fold increase in the number of antigen-specific antibody-secreting cells of both classes is observed in successfully immunized cultures, while essentially no antigen-specific antibody secretors are detected in the immunized bone marrow sample.

10. These results highlight the unique properties of peripheral lymphoid organ cultures with respect to both cell composition and the ability to respond to antigen stimulation.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

5/19/08
Date

/Andrea Bottaro/
Andrea Bottaro, Ph.D.

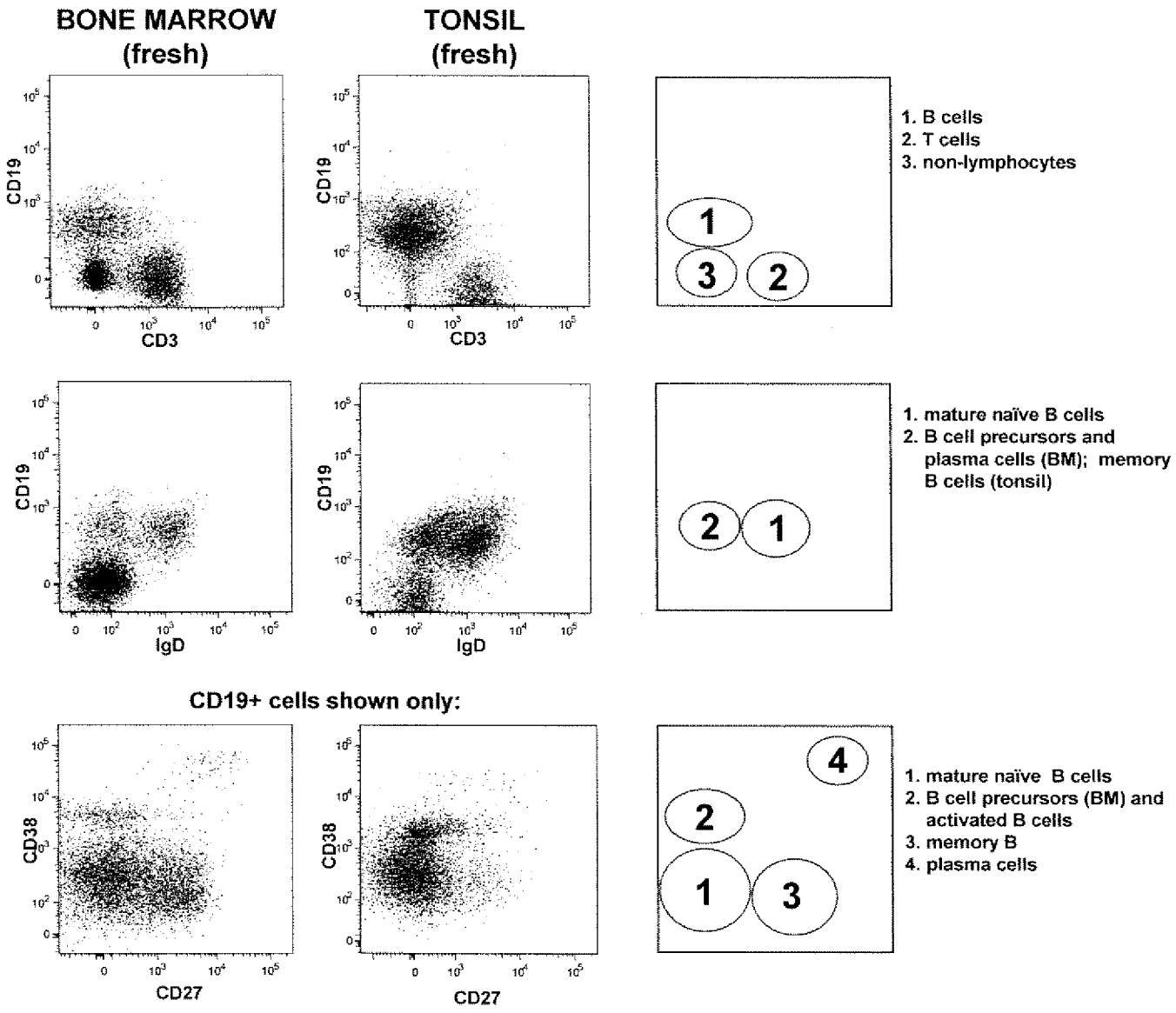

Figure 1

EXHIBIT 1

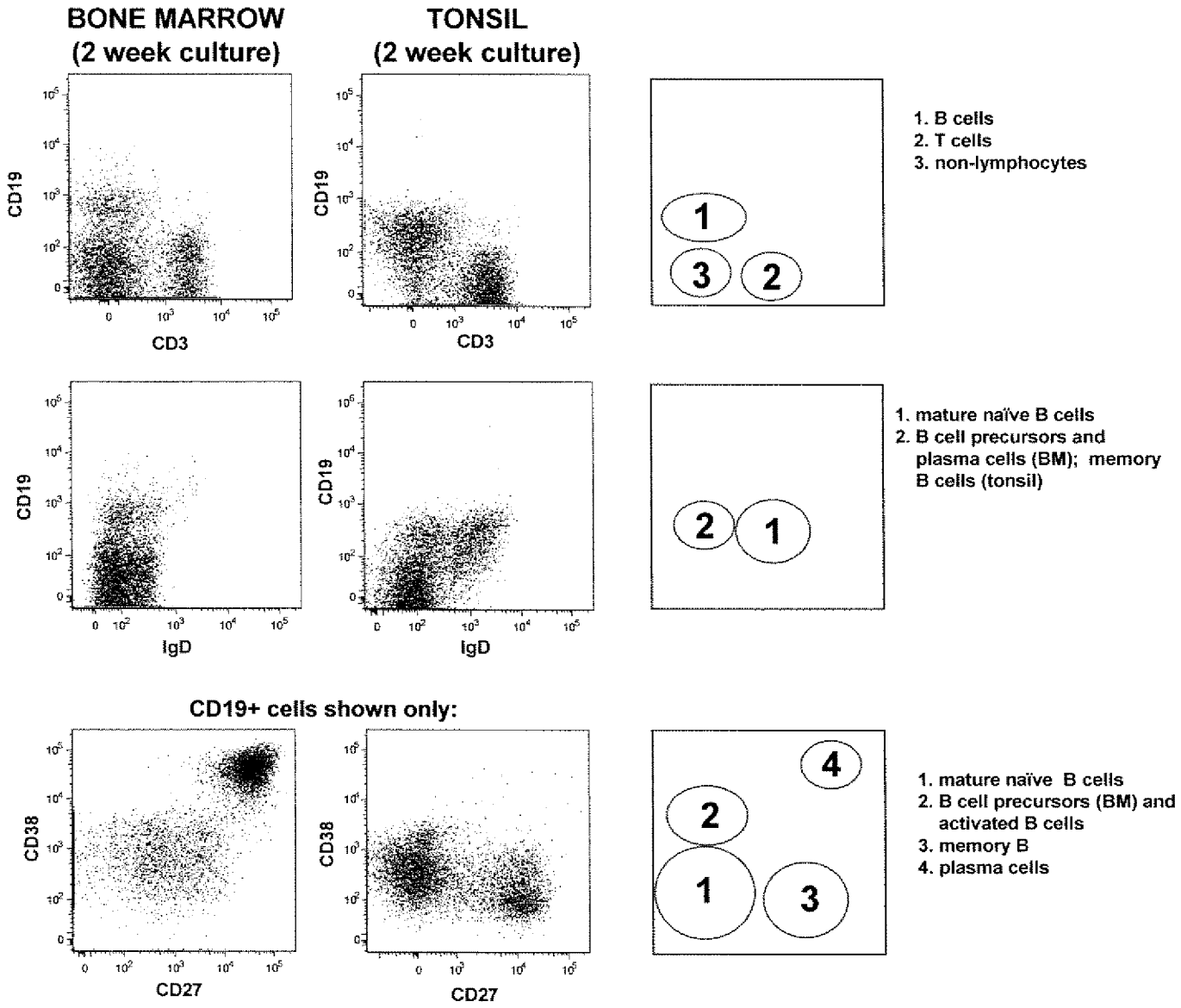
BONE MARROW
(2 week culture)
TONSIL
(2 week culture)
CD19
CD3
1. B cells
2. T cells
3. non-lymphocytes
IgD
1. mature naïve B cells
2. B cell precursors and plasma cells (BM); memory B cells (tonsil)
CD19+ cells shown only:
CD38
CD27
1. mature naïve B cells
2. B cell precursors (BM) and activated B cells
3. memory B
4. plasma cells

Figure 2

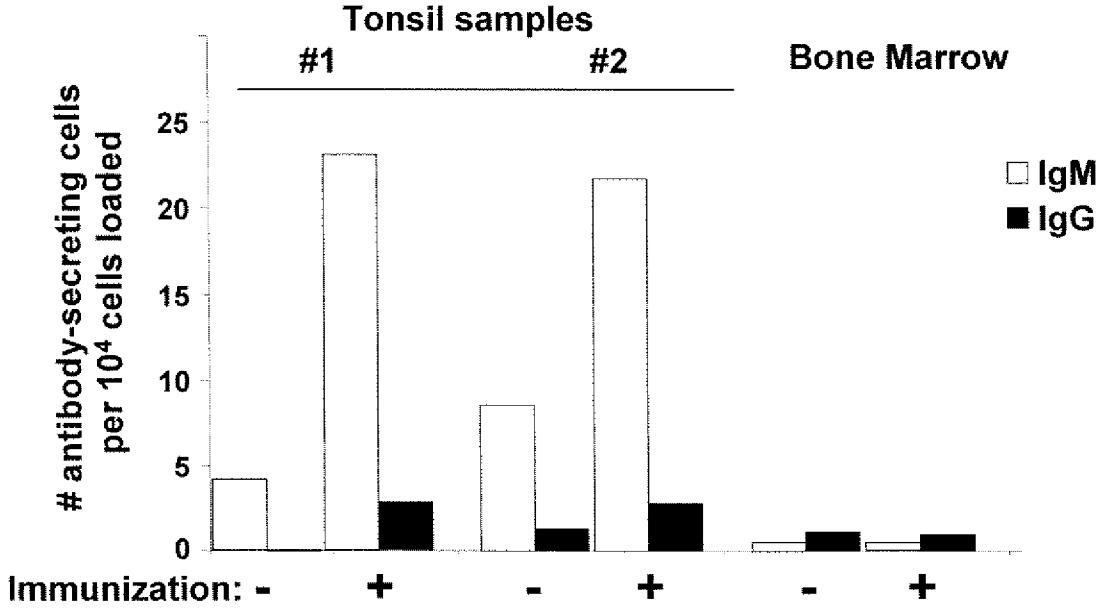
Tonsil samples
#1
#2
Bone Marrow
antibody-secreting cells per 10^4 cells loaded
25
20
15
10
5
0
IgM
IgG
Immunization: - + - + - +

Figure 3